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The catabolite activator protein stabilizes its binding site in the *E. coli* lactose promoter

Henry DeGrazia, Saras Abhiraman and Roger M.Wartell

Schools of Applied Biology and Physics, Georgia Institute of Technology, Atlanta, GA 30332, USA

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ABSTRACT

The effect of catabolite activator protein, CAP, on the thermal stability of DNA was examined. Site specific binding was studied with a 62 bp DNA restriction fragment containing the primary CAP site of the E. coli lactose (lac) promoter. A 144 bp DNA containing the lac promoter region and a 234 bp DNA from the pBR322 plasmid provided other DNA sites. Thermal denaturation of protein-DNA complexes was carried out in a low ionic strength solvent with 40% dimethyl sulfoxide, DMSO. In this solvent free DNA denatured below the denaturation temperature of CAP. The temperature stability of CAP for site specific binding was monitored using an acrylamide gel electrophoresis assay. Results show that both specific and non-specific CAP binding stabilize duplex DNA. Site specific binding to the 62 bp DNA produced a 13.3°C increase in the transition under conditions where non-specific binding stabilized this DNA by 2-3°C.

INTRODUCTION

The catabolite gene activator protein, CAP, in a complex with its cofactor cAMP, regulates transcription initiation of many Escherichia coli operons. This regulation is brought about by the binding of CAP-cAMP to specific DNA sites within catabolite sensitive promoters (1). The complex of CAP-cAMP and its promoter site generally enhances mRNA synthesis by RNA polymerase. The mechanism by which CAP-cAMP enhances transcription is not completely understood. DNA binding sites of CAP-cAMP are approximately 20 bp in length. Their location relative to the startpoint for transcription varies among different promoters. For the gal promoter, CAP-cAMP binds to a sequence between -25 and -50 (2). The lac promoter binding site is between -54 and -72 (3,4,5), and for the ara P_c promoter the CAP-cAMP binding site is between -78 and -107 (6). This diversity in the location of the CAP-cAMP binding sites relative to the startpoint suggests that the influence of the CAP-DNA complex on the RNA polymerase open promoter complex is variable. The ability of CAP-cAMP to act as a repressor of transcription for some promoters (7) must also be considered in deducing the mechanism(s) of CAP-cAMP's enhancement of

transcription.

Two models were initially proposed to explain the activation mechanism of CAP-cAMP. These models are not mutually exclusive. One model proposed that CAP-cAMP, when bound to DNA, interacts directly with RNA polymerase (8). A second model suggested that CAP-cAMP binding destabilizes the RNA polymerase binding site (9,10). Recent studies suggest a third model to explain CAP activation. Experiments indicate that CAP-cAMP stimulates mRNA expression of the gal and lac operons by blocking RNA polymerase from a strong binding but weak transcription site, thus allowing polymerase to bind a nearby productive initiation site (11,12,13).

The possibility that CAP-cAMP may act as a destabilizing protein in its site specific binding mode was examined by Unger et al. (14). A 301 bp fragment containing the lac promoter region was denatured in the absence and presence of CAP-cAMP. In 5 mM Na^+ a derivative melting curve of the 301 bp DNA alone shows two cooperatively melting peaks at 61.9°C and 66.4°C. The presence of CAP-cAMP resulted in the appearance of an additional small melting peak with a T_m of 64.4°C. This result suggested that site specific binding of CAP-cAMP stabilizes rather than destabilizes its binding region. The interpretation of the results with the 301 bp DNA are complicated however by the uncertain temperature stability of CAP, and the presence of many non-specific DNA sites and a secondary CAP binding site. Jensen and Von Hippel (14) have documented an example of a protein, ribonuclease A, which acts as a DNA melting protein at low temperatures, and becomes a DNA stabilizing protein after being heated to 55-60°C.

In order to help elucidate the thermodynamic binding properties of native CAP, we examined non-specific and site specific CAP binding to short DNA fragments. A 62 bp DNA containing the primary lactose promoter CAP site was employed. Figure 1 illustrates this fragment and the location of the CAP site. A 234 bp Hae III restriction fragment from pBR322 and a 144 bp DNA from the lactose promoter region were also examined. The influence of solvent, temperature and the presence of DNA on the binding activity of CAP was evaluated using a gel electrophoresis assay. Melting curve experiments were carried out in a solvent in which the transition midpoint temperature, T_m, of the 62 bp DNA was below the denaturation temperature of free CAP-cAMP. The melting curve studies indicated that site specific CAP-cAMP binding increases the stability of the 62 bp DNA by 13.3°C under conditions where non-specific binding stabilizes this DNA by 2-3°C. These results are only consistent with CAP-cAMP being a DNA stabilizing protein in its site specific binding mode.

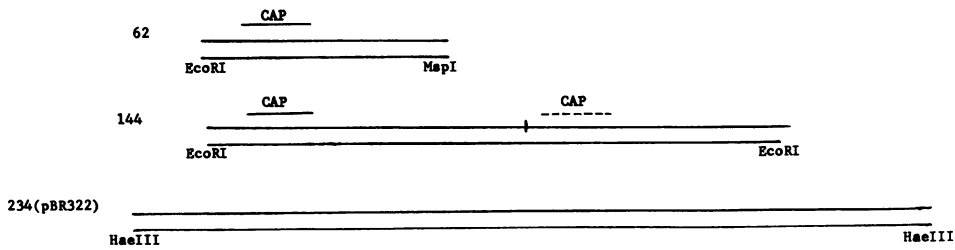

Figure 1. DNA restriction fragments used in this study. CAP sites are indicated for the 62 bp and 144 bp DNAs. Solid line underlining 'CAP' designates the primary functional site. The dashed line corresponds to a secondary CAP site located to the right of the major transcriptional startpoint (vertical slash).

MATERIALS AND METHODS

CAP isolation

CAP was isolated from an E. coli strain containing the plasmid pHA7 (16). This plasmid was constructed from an in vitro recombination of the plasmid pBR322 and the crp gene of E. coli. It was graciously provided by Hiroji Aiba. CAP was purified using a modification of the method of Boone and Wilcox (17). This method involved four steps. In step 1 a crude cell-free extract was prepared from 70 gm of frozen cells containing pHA7. A 10 ℓ. growth of pHA7 cells was disrupted in a French pressure cell at 10,000 psi followed immediately by centrifugation of the extract at 30,000 rpm for 90 min in a Beckman Ti-42.1 rotor. In step 2 the supernatant from step 1 was loaded onto a phosphocellulose column. We modified this step by employing a batch loading procedure.

Preequilibrated phosphocellulose was mixed with supernatant from step 1 by gentle stirring for 20 min. This mixture was allowed to settle and the top most layer of supernatant was removed. The phosphocellulose slurry was then loaded into the column. The remainder of this step was carried out as described by Boone and Wilcox (17). Active fractions from step 2, as measured by cAMP binding, were pooled and loaded onto a hydroxyappatite column (step 3). Active fractions from this column were collected, dialysed twice with 2 ℓ. of buffer C (20mM KPO_4, pH = 6.5, 1mM EDTA, 0.1 M NaCl) and loaded onto the final column. In this fourth step we substituted a denatured DNA-cellulose column for a DNA-DEAE-cellulose column. CAP was eluted using a pH jump from 6.5 to 8.0. Two 5 ml fractions were found to contain CAP at about 2 mg/ml each.

CAP concentrations were based on absorbance measurements using an

extinction coefficient of $\varepsilon_{280} = 3.5 \times 10^4\ M^{-1}\ cm^{-1}$, and confirmed using the cAMP binding assay (18). CAP was judged to be pure based on results from a 10% polyacrylamide gel containing SDS. The position of the isolated CAP in the gel was identical to a sample generously provided by A. Revzin. A single band was seen with 200 ug of protein loaded on the gel. CAP's ability to specifically bind to its DNA site was measured by the polyacrylamide gel electrophoresis assay of Garner and Revzin (19). About 25% of the CAP dimers was active for site specific binding in our preparation. This was similar to the value commonly found by others (19,20). CAP was stored in buffer C at 4^oC, and at -20^oC in 0.2M NaCl, 0.02M Tris (pH8 at 22^oC), 0.1mM EDTA with 50% glycerol. Under both conditions there was no loss of activity over several months.

DNA Fragments

The 234 bp fragment was purified from Hae III digested pBR322 and was a generous gift of A. S. Benight. The 144 bp DNA fragment from the lactose promoter and the 62 bp restriction fragment obtained from it, were isolated from the plasmid pRMW27 (21) which has two copies of the 144 bp lac DNA cloned into the Eco RI site of pVH51 DNA. The plasmid was maintained and grown in the E. coli strain MO. Purified plasmid was obtained from a modification of published procedures of Hardies and Wells (22). Following chloramphenicol amplified cell growth, lysis was carried out with lysozyme, Brij-58 and sodium deoxycholate. This procedure and the ensuing treatment with ribonuclease and phenol were similar to the published procedures. After phenol was removed by ether extraction and dialysis, the plasmid DNA was centrifuged to equilibrium in an ethidium bromide-CsCl gradient. The supercoiled DNA band was removed and the ethidium bromide extracted with isoamyl alcohol. The DNA was then dissolved and centrifuged in plain CsCl (ρ = 1.710 gm/ml). This second equilibrium centrifugation in CsCl alone significantly optimized the Eco RI digestion of the plasmid DNA. Ethidium bromide-CsCl runs did not remove some uncharacterized Eco RI inhibitor. The yield of pure plasmid varied from 2-3 mg/liter of cell growth.

The purified plasmid was cleaved with Eco RI. The 144 bp DNA was separated from the vector DNA by selective polyethylene glycol (PEG) precipitation in the presence of gelatin. The PEG step resulted in a considerable reduction of contaminating vector DNA. The lac fragment and the remaining vector were phenol extracted and separated by RPC-5 column chromatography. 7% polyacrylamide gel electrophoresis of 3 ug of the 144 bp DNA verified that it was purified to homogeniety.

The 62 bp DNA was prepared by cleaving the 144 bp DNA with Msp I and separating it from the other fragment by RPC-5 chromatography. The cleaved 144 bp DNA was phenol extracted prior to loading it on the RPC-5 column. Polyacrylamide gel electrophoresis was employed to verify the clean separation of the two DNAs indicated by the uv-absorbance elution profile. From 2 mgs of the 144 bp DNA, we recovered 0.74 mgs of the 62 bp DNA and 0.9 mgs of the 80 bp DNA.

Sample Preparation

Most of the melting curves were obtained using a solvent containing 1mM NaCl, 2mM Tris (pH = 8.0), 0.06mM EDTA and 40% dimethyl sulfoxide, DMSO. The DMSO was obtained from Aldrich Chem. Co. as 'ultrapure' spectrophotometric grade. When present, cAMP (Sigma Chem. Co.) was at a concentration of 10uM. Samples were prepared by first dialysing the DNA into 1.7mM NaCl, 3.3mM Tris, and 0.1mM EDTA. This solution was placed in ice, and DMSO was added in 20 ul amounts. Serial additions were continued until a final concentration of 40% DMSO was reached. If DNA solutions were not cooled before DMSO was added irreproducible results were obtained. This was due to heat generated from the exothermic reaction of DMSO and water, which apparently melted portions of the DNA. For samples with CAP, the CAP was added to the DNA in a solvent containing 0.1M NaCl and 3mM Tris. The mixture was then dialysed to the low salt solution and DMSO added as before. When cAMP was present it was added prior to the DMSO additions. A few experiments were carried out using 5mM sodium cacodylate (pH = 7.0) or 0.1M KCl + 0.04M Tris (pH = 8.0). CAP was dialysed directly into these solvents with or without DNA.

Thermal Denaturation

Absorbance-temperature profiles were measured at 268 nm using a Beckman Acta MVI spectrophotometer. Sample volumes were 0.7 ml. They were heated at a rate of about $7^{\circ}C$/hour using a Lauda circulating bath. Further details of the experimental system have been previously given (23). The digital absorbance vs. temperature data was smoothed using the Savitsky and Golay method (24). Derivative melting curves were obtained by normalizing the absorbance change from 0 to 1.0, and taking the derivative of the smoothed melting curve. $d\theta/dT$, the change in the fraction of broken base pairs was plotted versus temperature. The T_m of a derivative melting curve was defined as the temperature at which $d\theta/dT$ was a maximum. Two or three transitions were taken to verify reproducibility.

Binding Assay

An assay involving polyacrylamide gel electrophoresis was employed to

determine the effect of solvent, temperature, and the presence of DNA on site specific CAP binding. Previous studies have demonstrated that CAP-cAMP lowers the mobility of short DNA fragments which contain specific CAP sites (19,20). 20 ul samples were taken from a solution of 0.5-5 uM CAP in a given solvent and at a specified temperature. In some experiments DNA was also present in the solution. After cooling the sample to 25°C, 2-3 ul of the 62 bp. DNA fragment in a similar solvent was added to give a final concentration ratio of one 62 bp. DNA molecule per four CAP dimers. This mixture, at a total volume of about 25 ul., was incubated for 15 minutes at room temperature, and then loaded onto a 7.5% polyacrylamide gel (46:1, acrylamide: bisacrylamide). Generally 3 ul of 0.3% bromphenol blue solution with 30% glycerol was added as a visual marker just prior to loading the samples. Omitting this dye solution gave identical results. Ethidium bromide staining was employed to visualize the DNA bands in the gel. Some experiments involved ^{32}P labeled 144 bp DNA as the probe (generously supplied by D. Dripps). For these experiments, autoradiography was employed to visualize DNA bands. The electrophoresis buffer contained 0.089M Tris, (pH8.5), 0.089M sodium borate, 0.1mM EDTA, and 10uM cAMP. Determinations of CAP thermal stability were made employing heating conditions identical to those used for the DNA melting curves. In some instances CAP stability measurements were made from the same CAP-DNA solutions being monitored for DNA melting. The results were the same for both cases.

RESULTS

CAP Binding to DNA in DMSO

An assessment of the effect of CAP-cAMP on DNA melting requires that CAP maintain its native binding activity through the melting region. We therefore sought a solvent in which CAP remains active for site specific binding at a temperature well above the T_m of the DNA alone. After investigating several solvents, it was determined that the low ionic strength solvent with 40% DMSO met these criteria. In this solvent, the 62 bp DNA alone has a melting temperature of 31.7°C (see below). Figure 2 shows the temperature stability of free CAP-cAMP for site specific binding. CAP-cAMP was removed from the 40% DMSO solvent at various temperatures, allowed to cool to 25°C, and challenged with the 62 bp DNA to determine whether it was irreversibly denatured. Below 41°C, CAP-cAMP retained its ability to alter the mobility of the probe DNA. The change in mobility was the same as that observed for CAP in the commonly used 100mM KCl solvent (not shown). Above

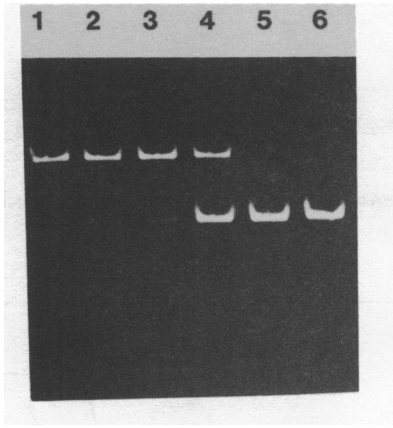

Figure 2. Gel assay of DNA specific binding after heating CAP-cAMP to various temperatures in 40% DMSO solvent. 10uM cAMP was also present. CAP concentration was 4.3 x 10^{-7}M. 20 ul of CAP was removed and 62 bp DNA added to generate 4/1 ratio of CAP/DNA (See Binding Assay). Lanes from left to right in ^{o}C; 25,30, 35,41,43,45. Lower band is free 62 bp DNA,upper band corresponds to CAP-DNA complex.

41^{o}C, the site specific binding of CAP-cAMP for the 62 bp DNA immediately decreases. It was also demonstrated that cAMP is required for site specific binding of CAP in 40% DMSO. CAP at a concentration of 0.6uM had no effect on the mobility of 0.15uM of the 62 bp DNA. Adding 10uM cAMP to an equivalent sample resulted in the DNA moving with the mobility characteristic of the site specific complex. Titration experiments showed that the same ratio of CAP to DNA was required to eliminate the free DNA band in 40% DMSO as in the 0.1M KCl solvent. Thus CAP shows site specific binding in the 40% DMSO solvent at a temperature 10^{o}C higher than the T_m of the 62 bp DNA.

The irreversible denaturation of CAP above 41^{o}C was correlated with a marked increase in light scattering from CAP-cAMP solutions. The apparent absorbance increased by 3-4 fold at 280 nm. This correlation did not occur when DNA was present either in the DMSO solvent or purely aqueous solvents. This is illustrated for a 5mM Na^{+} solvent in which CAP alone denatured around 48^{o}C in agreement with Takahashi et al. (25). When DNA was added CAP stability increased. Figure 3 illustrates the temperature stability of CAP-cAMP in a 5mM Na^{+} solution containing the 144 bp DNA. Below the midpoint of melting of the CAP-DNA complex (58^{o}C), CAP-cAMP retains its ability to bind in a site specific manner (Fig. 3a). Above 58^{o}C the 144 bp DNA is partially melted (Fig. 3b) and CAP-cAMP binding decreases. However no increase in light

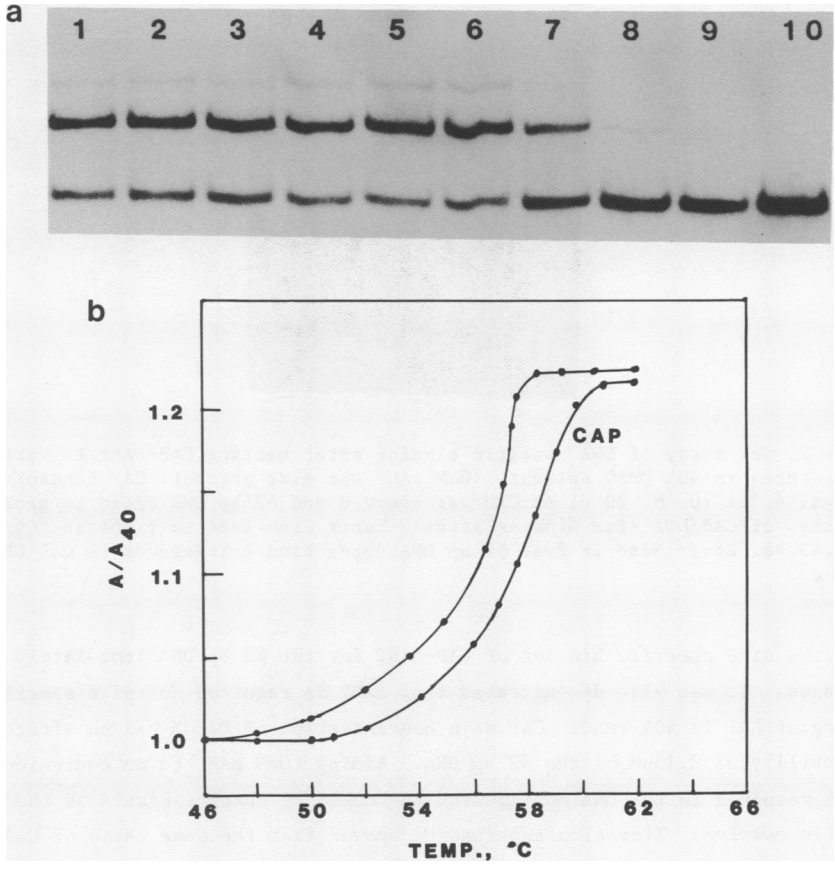

Figure 3. (a) Assay monitoring thermal stability of CAP-cAMP in the presence of 144 bp DNA in 5mM Na^+ . CAP/DNA ratio was 4/1 before adding the probe DNA. Aliquots were removed at specified temperatures and mixed with ^{32}P labelled unheated 144 bp DNA. This DNA was of low specific activity,and changed the CAP/DNA ratio to 3.2/1. This explains the presence of the free DNA band at low temperatures. Lanes 1,2,... 10 correspond to temperatures (°C); 33.,35.5,41.,45.,51., 55.7,58.4,60.1,66.,70. Bottom band is free DNA. Faint top band is DNA with two bound CAP. (b) Melting curves of 0.3uM 144 bp DNA alone (-•-•-) and with 4/1 ratio of CAP/DNA (labeled CAP). Solvent was 5mM Na^+ and contained 10uM cAMP. The absorbance relative to the 40°C value is plotted vs temperature.

scattering was observed even after heating the CAP-cAMP-DNA solution to 90°C. Thus the absence of light scattering does not necessarily imply that CAP retains its native binding affinity.

The Influence of Non-Specific CAP Binding on DNA Stability

The effect of non-specific CAP binding on DNA stability was examined in 40% DMSO with the 62 bp lac DNA and the 234 bp DNA from pBR322. The 234 bp

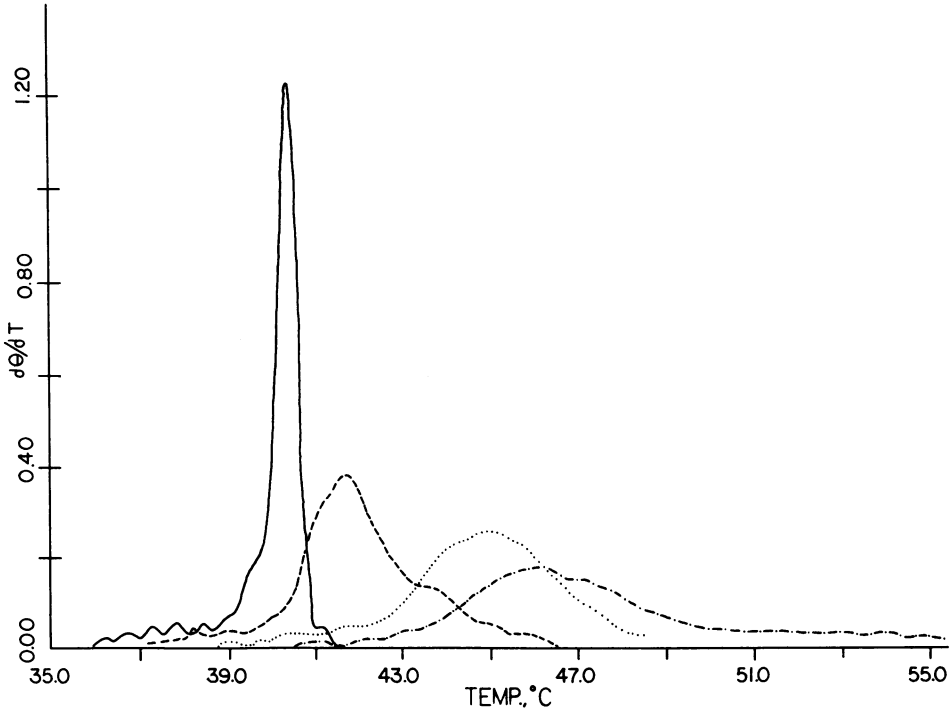

Figure 4. Derivative melting curves of 234 bp DNA in 40% DMSO solvent. Solid line has no CAP. Dashed line has a 7/1 ratio of CAP/DNA, dotted line has a 14/1 ratio of CAP/DNA, and dot-dash line has a 21/1 ratio of CAP/DNA. 10uM cAMP was in the solvent.

DNA does not have strong CAP-cAMP binding sites as indicated by the electrophoresis assay. Figure 4 shows derivative melting curves of the 234 bp DNA at several ratios of CAP-cAMP to DNA. Without CAP, the 234 bp DNA has a T_m of 40.4°C (± cAMP). Increasing amounts of CAP lead to a progressive increase in the transition temperature, a decrease in peak height and an increase in width of the transition. At 21 CAP-cAMP added per DNA the transition midpoint is increased by 5.4°C to 45.8°C and the width is increased by 5.6°C (full width at 0.5 maximum height).

Since the melting temperature of the 234 bp DNA saturated with CAP-cAMP is above the CAP denaturation temperature (41°C), some inactivation of CAP may occur prior to DNA melting. This was examined using the electrophoresis assay. Aliquots of the CAP-234 bp DNA solution were heated to various temperatures, cooled to 25°C, and then added to the 62 bp DNA. Site specific binding was observed until 45-46°C. Thus CAP bound to the 234 bp DNA was not

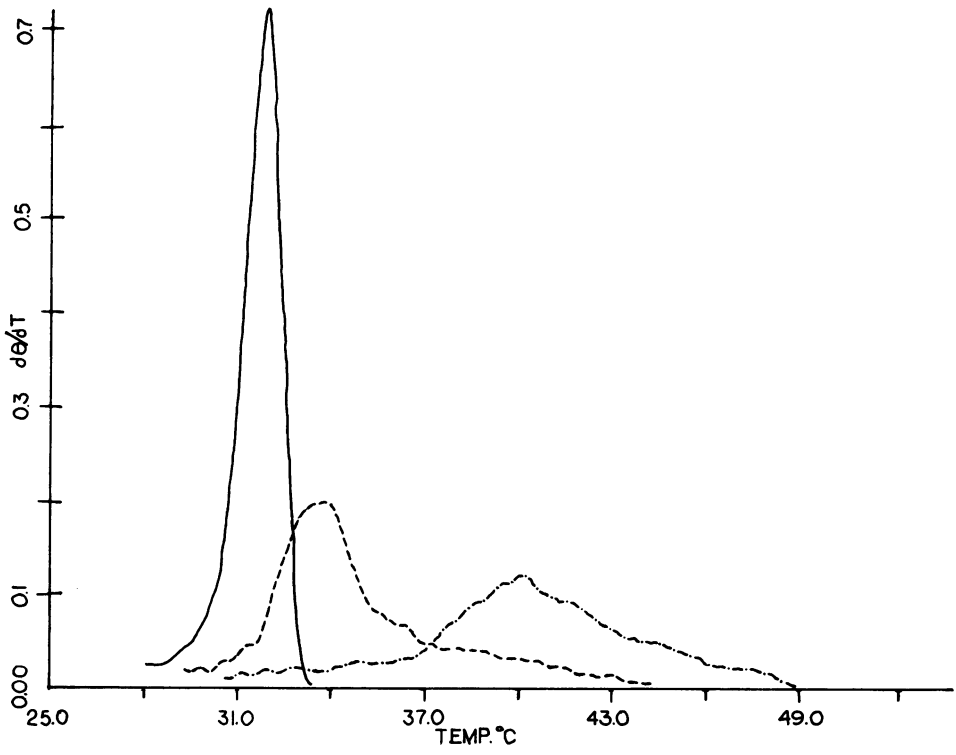

Figure 5. Derivative melting curves of 62 bp DNA in 40% DMSO solvent. Solid line has no CAP, dashed line has a 2.6/1 ratio of CAP/DNA, and dash-dot line has a 8/1 ratio of CAP/DNA.

irreversibly denatured up to the melting region.

The effect of non-specific CAP binding on the stability of the 62 bp lac DNA was also examined. In the absence of cAMP, CAP does not show site specific binding. Figure 5 shows derivative melting curves for the 62 bp DNA with increasing concentrations of CAP. Table I summarizes these results.

The effect of CAP alone on the 62 bp fragment melting curve was similar to that observed for the 234 bp DNA. The T_m of the transition increased with increasing CAP, and the transition cooperativity was reduced. Fewer CAP molecules were needed to saturate the shorter 62 bp DNA. The melting temperature of the 62 bp DNA fragment was increased to 40.1°C with eight CAP per DNA molecule. Since this DNA transition occurred below the temperature of CAP denaturation, the 8.4°C increment is not subject to uncertainties of CAP inactivation. Unlike poly(dA-dT), there is little or no indication of a bi-phasic transition at low CAP/DNA ratios (25). This can be explained by the

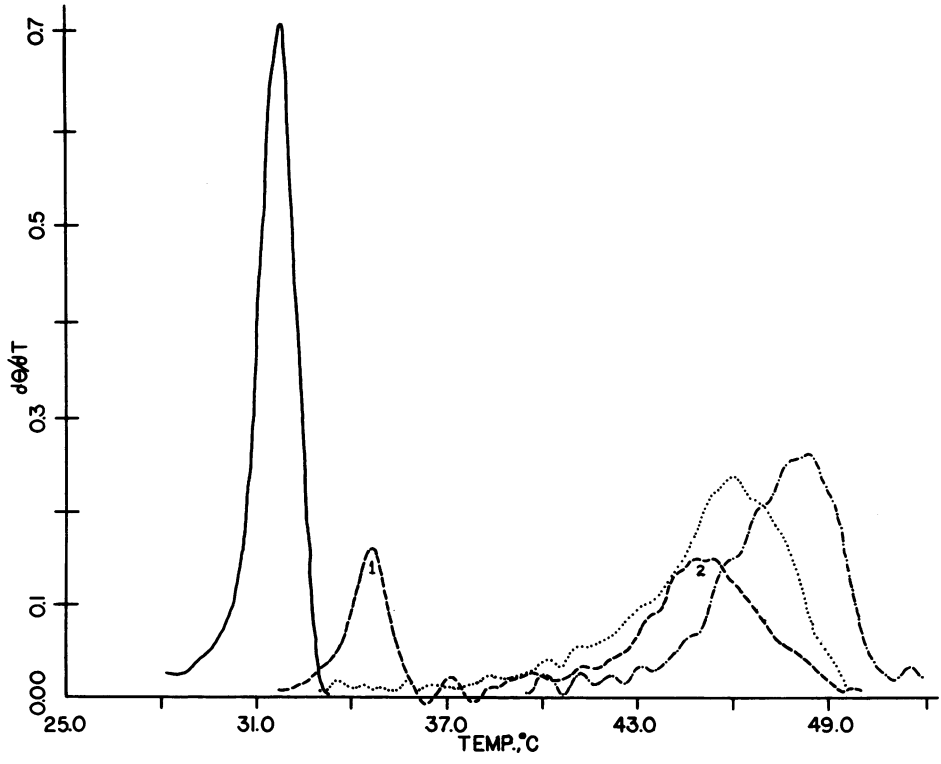

Figure 6. Derivative melting curve of 62 bp DNA with and without CAP-cAMP in 40% DMSO solvent. 62 bp DNA alone is solid line.Dashed line has 2.8/1 of CAP-cAMP/DNA,dotted line has 5.3/1 of CAP-cAMP/DNA,and dash-dot line has 8/1 of CAP-cAMP/DNA. 10uM cAMP was in the solvent.

short lengths of the DNAs, which are comparable to the cooperativity length of DNA melting. Since CAP binds strongly to DNA ends (27), it will initially bind non-cooperatively to all DNAs increasing their overall stabilities.

The effect of specific CAP binding on the 62 bp DNA and 144 bp DNA

The results described earlier demonstrated that CAP with cAMP exhibits site specific binding to the 62 bp DNA. Figure 6 shows the influence of CAP-cAMP on the melting transition of the 62 bp fragment in 40% DMSO. In the absence of CAP, the 62 bp DNA has a T_m of 31.7°C. The addition of 2.8 CAP dimers per DNA with cAMP results in a biphasic melting curve. The first subtransition has its peak at 34.7°C and a width of 1.6°C. The second subtransition has a peak at 45°C and a width of 4.1°C. The integrated areas of subtransition 1 vs. subtransition 2 are in the ratio of 0.3 to 0.7. The

TABLE I

Summary of results from derivative melting curves shown in figures 4, 5,and 6. W is the width of the transition or subtransition at half the maximum peak height.

DNA	cAMP	[CAP] / [DNA]	T , C	W , C
234 bp	+	0	40.4	.9
	+	7	41.8	3.4
	+	14	45.0	5.5
	+	21	45.8	6.5
62 bp	-	0	31.7	1.6
	-	2.6	33.8	3.0
	-	8.0	40.2	5.8
62 bp	+	0	31.7	1.6
	+	2.8	34.7,45.0	1.6,5.1
	+	5.3	46.0	5.3
	+	8.0	48.1	5.0

simplest interpretation of this ratio is that 70% of the 62 bp DNAs were stabilized 13.3°C, and 30% of the DNAs were stabilized 3°C. An analysis of the CAP-cAMP-DNA complexes formed at 2.8 CAP dimers per DNA indicates that subtransition 2 corresponds to site specific binding of CAP-cAMP and subtransition 1 corresponds to non-specific binding. Measurements discussed earlier showed that only 25% of CAP dimers are capable of site specific binding. Thus for 2.8 CAP dimers per DNA there are 0.7 CAP dimers per DNA capable of site specific binding. Assuming an equilibrium binding constant in 40% DMSO similar to that measured in other low ionic strength solvents (10^{11} M^{-1}), one can expect all of the CAP capable of site specific binding to be bound. Site specific binding can account for shifting 70% of the DNA to the higher melting subtransition. The remaining CAP can bind non-specifically to all DNA fragments. The 3°C increase observed for the first subtransition is similar to the 2.1°C increase observed for the non-specific binding of 2.6 CAP dimer per 62 bp DNA. (See Fig. 5 and Table I.)

Higher ratios of CAP-cAMP dimer to 62 bp DNA resulted in monophasic melting transitions (Fig. 6). The integrated areas of the transitions were the same as the combination of the two peaks observed at 2.8 CAP dimers/DNA. At 5.3 CAP dimers per DNA, the T_m is increased by 14.3°C. It is further increased to 48.1°C with 8 CAP dimers per DNA. The absence of a low temperature subtransition at CAP levels which saturate the specific DNA sites, and the equivalence in the transition areas are consistent with the earlier subtransition assignments. The electrophoresis assay verified that CAP-cAMP

retained its specific binding activity up to the melting region.

The influence of CAP-cAMP on the stability of the 144 bp DNA was also examined in 40% DMSO. In addition to the primary CAP site, the 144 bp DNA has a second site with a binding constant about 40 fold weaker than the primary site (26). The results of this study were similar to Fig. 6 (not shown). The 144 bp DNA alone melted with a T_m of 34.9°C. With one or two CAP dimers per DNA two melting peaks were observed at about 37-38°C and 42°C. The ratio of four CAP dimers per DNA gave one transition at 42.2°C.

DISCUSSION

The melting curve results show that CAP-cAMP stabilizes duplex DNA in both non-specific and site specific complexes. Saturation of the 234 bp DNA increased the melting temperature by 5.4°C. An 8.4°C increase was observed for the saturation of non-specific sites on the 62 bp DNA. The larger increase for the 62 bp DNA may be due to the more cooperative binding of CAP without cAMP (18), and/or the larger relative influence of the fragment ends. CAP has been observed to frequently bind to the ends of DNA fragments (27). The biphasic transitions observed at low ratios of CAP-cAMP to the 62 bp DNA (Fig. 6) and the 144 bp DNA can be explained by a combination of non-specific and site-specific binding. Only 25% of our CAP preparation was active for site-specific binding. When the number of active CAP molecules is less than the number of DNA molecules, a fraction of the DNA will interact with the inactive CAP. The low temperature subtransitions appear to be due to the inactive CAP molecules which are incapable of site-specific binding, but still capable of non-specific binding. The high temperature subtransition results from site-specific CAP-cAMP binding.

We theoretically asked the question, by how much would CAP-cAMP have to stabilize its 20 bp site in order to increase the 62 bp DNA melting curve by about 14°C? The helix-coil transition theory of DNA was employed (28,29). This calculation is not rigorously related to the experimental melting curves of the 62 bp DNA since a number of uncertain assumptions are necessary. DNA melting parameters are not known for the 40% DMSO solvent. Parameters determined in 0.1M NaCl were used. σ, the stacking cooperativity parameter was 4.5×10^{-5}, and $T_{GC} - T_{AT}$, the T_m difference between pure A.T and G.C DNAs was 43°C (29). The calculation also assumes that CAP-cAMP binding is approximately temperature independent. This latter assumption should be valid for temperatures below the free CAP denaturation temperature. Above the free CAP denaturation temperature, the situation is a complex one depending on the

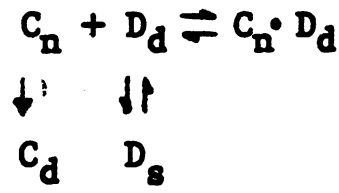

Figure 7. Major reactions governing the effect of CAP-cAMP on DNA melting. D_d and D_s correspond to duplex and single stranded DNA. C_n and C_d correspond to native (active) and denatured (inactive) CAP-cAMP. $C_n \cdot D_d$ correspond to the protein DNA complex.

rates of denaturation of free CAP, and the dissociation and reassociation rates of CAP to DNA (see below). Given these caveats it is of interest to note that increasing the melting temperatures of all base pairs in the region -50 to -69 by 35° C increased the T_m of the 62 bp DNA transition by 13.5°C. The predicted width of the transition broadened and the peak height decreased as was experimentally observed. Repeating this calculation for the 144 bp DNA resulted in an increase in T_m by about 7°C with similar changes in transition width and peak height. These theoretical results are in general accord with the melting curve data, and suggest that under the solvent conditions examined CAP-cAMP stabilizes its site by about 35°C.

Under physiological conditions it is likely that CAP-cAMP also acts as a DNA stabilizing protein. This is indicated by the results obtained by Kolb and Buc (30). CAP-cAMP when specifically bound to its lac site in supercoiled DNA in 100 mM KCl does not significantly unwind DNA. Although this does not establish CAP as a stabilizing protein, it undermines the notion that it is a melting protein. A direct assessment of CAP-cAMP's effect on DNA stability cannot unfortunately be made in 0.1-0.2M Na^+. If free CAP's denaturation temperature, T_D, is above the free DNA melting transition, T_m, one can determine if CAP is a stabilizing or melting protein. When T_D is less than the free DNA T_m (which occurs for the 62 bp DNA in 0.1M Na^+) the results are difficult to interpret. Figure 7 illustrates the major reactions governing how CAP will influence DNA stability. The rates of CAP denaturation, CAP-DNA on and off rates, and base pair opening and closing at the CAP site all influence the outcome. Once the protein DNA complex is heated past T_D, the potential exists for CAP to denature when it dynamically dissociates from DNA. As the temperature increases, one can expect the rate constant for CAP denaturation to increase. Base pair opening will also increase and will deplete duplex DNA sites. Since CAP binds weakly to single stranded DNAs

(18), the amount of free CAP, susceptable to rapid denaturation, will rise. For a situation where $T_m > T_D$, CAP may become inactive as soon as base pair opening becomes significant. The extent to which CAP stabilizes a DNA will be limited.

This appears to be the case in the 5mM Na^+ solvent. The T_m of the free 144 bp DNA (56.4°C) is greater than the free CAP denaturation temperature (48°C). CAP does not denature as long as the DNA remains a duplex. However, when the temperature exceeds the T_m, the large value of $T-T_D$ results in a high denaturation rate of CAP. A 1.6°C increase in the DNA's thermal stability is observed. This is less than the 7.3°C increase obtained for the same CAP-DNA ratio in the 40% DMSO solvent. In the latter solvent the free DNA T_m is less than T_D. Another result tends to corroborate the above conclusion. We examined the effect of CAP-cAMP on the stability of poly d(G-C) poly d(G-C) in the 40% DMSO solvent. This DNA polymer has a T_m of 56°C in this solvent. Saturating levels of CAP-cAMP (1 CAP dimer/12 base pairs) had essentially no effect on the transition T_m. The protein remained active for site specific binding (using the gel assay) until the DNA polymer reached the melting region. This behavior is qualitatively expected. Since T_m-T_D is large (15°C) CAP denatured as soon as base pair opening began to rise significantly. This did not occur for the non-specific binding of CAP to the 62 bp and 234 bp DNAs. Here $T_m<T_D$, and the stabilizing effect of CAP was observed.

The results and analysis indicate that site-specific CAP-cAMP binding stabilizes its promoter site. This physical behavior is consistent with CAP acting as a repressor by blocking RNA polymerase from strong initiation sites, and as an activator by displacing RNA polymerase from poor initiation regions to good initiation regions. The influence of CAP-cAMP on RNA polymerase may also involve other interactions as a result of DNA bending and protein-protein contacts. However the idea of a transmitted change in DNA stability has no experimental support.

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